Mitogen-Activated Protein Kinase/Extracellular Signal-Regulated Kinase Activation in Somatodendritic Compartments: Roles of Action Potentials, Frequency, and Mode of Calcium Entry

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Mitogen-activated protein kinase (MAPK) has been identified as a potential element in regulating excitability, long-term potentiation (LTP), and gene expression in hippocampal neurons. The objective of the present study was to determine whether the pattern and intensity of synaptic activity could differentially regulate MAPK phosphorylation via selective activation of different modes of calcium influx into CA1 pyramidal neurons. An antibody specific for the phosphorylated (active) form of MAPK was used to stain sections from hippocampal slices, which were first stimulated *in vitro*.

LTP-inducing stimulation [theta-burst (TBS) and 100 Hz] was effective in inducing intense staining in both dendritic and somatic compartments of CA1 neurons. Phosphorylation of MAPK was also induced, however, with stimulation frequencies (3–10 Hz) not typically effective in inducing LTP. Intensity and extent of staining was better correlated with the spread of population spikes across the CA1 subfield than with frequency (above 3 Hz). Experiments using inhibitors of NMDA receptors and voltage-sensitive calcium channels (VSCCs) revealed that, although MAPK is activated after both TBS and 5 Hz stimulation, the relative contribution of calcium through L-type calcium channels differs. Blockade of NMDA receptors alone was sufficient to prevent MAPK phosphorylation in response to 5 Hz stimulation, whereas inhibitors of both NMDA receptors and VSCCs were necessary for inhibition of the TBS-induced staining. We conclude that the intensity and frequency of synaptic input to CA1 hippocampal neurons are critically involved in determining the path by which second-messenger cascades are activated to activate MAPK.

Key words: long-term potentiation; hippocampus; ERK; activity-dependent; dendrite; soma; CA1

A defining feature of long-term potentiation (LTP) is its dependence on certain patterns of afferent stimulation. LTP is typically induced by trains of high-frequency stimulation (≥100 Hz) (Bliss and Lomo, 1973) or by brief, high-frequency bursts repeated at the theta rhythm (Larson et al., 1986); lower frequencies of stimulation (1–20 Hz) can result in short-term increases in synaptic strength [short-term potentiation (STP)] (Cummings et al., 1996) or decreases in synaptic strength [long-term depression (LTD)] (Dudek and Bear, 1992) (but see Thomas et al., 1998). On a molecular level, stimulus frequency-dependent differences in the induction of LTP, STP, and LTD have been associated with the concentration of intracellular calcium induced by the associated stimulus frequencies (Cummings et al., 1996), but the subsequent calcium-dependent reactions are only beginning to be identified and investigated. Related to the induction of the late-phase of LTP are experiments in culture showing that the mode of calcium entry, i.e., synaptic, through NMDA receptors or somatic, through voltage sensitive calcium channels (VSCCs) can determine which calcium-dependent signaling cascades and subsequent gene expressions are activated (for review, see Bading et al., 1993; Diesseroth et al., 1996; Ginty, 1997). Because firing frequency and the amplitude of the synaptic potential (proportional to stimulus intensity) are important factors in regulating dendritic and nuclear calcium dynamics (Nakazawa and Murphy, 1999), it is likely that they could similarly mediate plasticity and signaling to the nucleus.

Mitogen-activated protein kinases (MAPK), particularly the extracellular signal regulated kinase (ERKI/II), can respond to stimulation by neurotrophins, neuromodulators, and intracellular calcium and can lead to the phosphorylation of cAMP response element-binding protein (CREB) and other transcription factors to mediate gene expression (for review, see Rosen et al., 1994; Xia et al., 1996; Impey et al., 1999). In addition, activation of MAPK has also been proposed to regulate LTP in CA1 of the hippocampus. Contributing to this view are numerous studies showing the following: (1) inhibitors of MAPK block LTP induction and/or its late-phase expression (English and Sweatt, 1997; Impey et al., 1998) (but see Liu et al., 1999; Winder et al., 1999; Kanterewicz et al., 2000); (2) glutamate and potassium are very

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effective in activating MAPK in cultured hippocampal neurons and in slices (Bading and Greenberg, 1991; Kurino et al., 1995; Baron et al., 1996); and (3) MAPK is activated both in slices and *in vivo* by LTP-inducing stimulation and certain learning paradigms (English and Sweatt, 1996; Atkins et al., 1998; Blum et al., 1999; Davis et al., 2000). Furthermore, because MAPK is localized in both cell bodies and dendrites of neurons (Fiore et al., 1993), it may also play a role in modulating cellular and dendritic excitability (Winder et al., 1999) or cytoskeletal function (Quinlan and Halpain, 1996). The potential for subcellular heterogeneity of MAPK activation via different types of stimuli, therefore, could be an important factor in initiating distinct forms of plasticity by different patterns of activation. In this study, we examined the dependence of MAPK phosphorylation on the frequency and intensity of synaptic activity and whether different modes of calcium entry (mediated via NMDA receptors vs VSCCs) or signaling pathways in spatially distinct cellular compartments contribute differentially to activate MAPK in CA1 neurons.

MATERIALS AND METHODS

Slice preparation and physiology. Hippocampal slices (400 μm) were prepared from hooded or albino Sprague Dawley rats between the ages of 5 and 12 weeks, although similar results were obtained with tissue from mice of different strains. Slices were cut on a vibraslicer in ice-cold artificial CSF (ACSF) containing (in mM): NaCl 124, KCl 4, NaH_2PO_4 1.25, $NaHCO_2$ 26, $CaCl_2$ 2, $MgCl_2$ 2, and glucose 10, bubbled with 95% O_2–5% CO_2. Slices were perfused at 1 ml/min in an interface chamber [Medical Systems/Haas (Greenvale, NY) top; larger synaptic potentials were observed using the Oslo/Fine Science Tools (Foster City, CA)-type interface chamber, but the design of the Haas chamber was better-suited for quick removal of slices with minimal mechanical manipulation]. Slices were maintained at 34°C because comparatively little staining for phospho-MAPK could be induced when slices were incubated at 28°C. Concentric bipolar stimulating electrodes (Frederick Haer & Co. Inc., Brunswick, ME) were placed in the stratum radiatum, and for recording of population-spikes, an ACSF-containing glass recording electrode was placed in stratum pyramidale. Unless stated otherwise, stimulation was delivered with an intensity of 140 μA and a duration of 50 μsec. In all experiments, slices were stimulated with 120 pulses, because fewer than 80 pulses did not reliably produce staining. Theta-burst stimulation (TBS) (Larson et al., 1986) consisted of 10 bursts of four pulses at 100 Hz, delivered at 5 Hz, given a total of three times, with a 15–30 sec interval. Because MAPK is rapidly dephosphorylated, slices were fixed in 4% paraformaldehyde 1–3 min after the last pulse of stimulation (less staining, in a perinuclear pattern, was observed when slices were fixed within 60 sec of stimulation); no attempt was made to determine whether LTP had been induced in each slice because of the requirement for rapid fixation. Tests of LTP induction were often used in other slices to determine wash-in of drugs, such as APV. Drugs were purchased from Tocris Cookson (Ballwin, MO).

Immunocytochemistry. Slices were fixed overnight and then cryoprotected with a 24 hr incubation in 10% sucrose–4% paraformaldehyde. Frozen slices were recut at 30 μm, and the resulting sections washed three times in Tris-buffered saline, incubated for 30 min in 50% ethanol, permeabilized with three 15 min washes in 0.3% Triton X-100 in 1% normal goat serum (NGS), and blocked for 1 hr with 3% NGS, 0.3% Triton X-100. Primary antibody to dually phosphorylated ERK I/II (Promega, Madison, WI) in 1% NGS was used at a 1:7000–1:10,000 dilution for 48 hr. Sections were washed, incubated with a biotinylated secondary antibody at 1:1000 for 90 min, washed again, incubated for 45 min with avidin-biotin complex (Vector Elite ABC; Vector Laboratories, Burlingame, CA), and then processed for DAB reaction product. To avoid possible saturation of the DAB reaction product, the antibody concentration was chosen to be sufficiently dilute to allow the reaction to proceed at a rate slow enough to permit cessation of the reaction before the point of maximum intensity of stain. The reaction product was specific for a MAPK- kinase (MEK)-dependent process because staining was essentially eliminated by 20 μM of the MEK1 and MEK 2 inhibitor U0126 (data not shown).

Images (4 or 20×) were acquired digitally on a microscope equipped with a SPOT digital camera (Diagnostic Instruments, Sterling Heights, MI), and subsequently analyzed with Metamorph software (Universal Imaging, West Chester, PA). The three most representative and/or most complete sections from each slice were chosen for analysis, and the average intensity was determined in 20 × 20 pixel areas in both stratum radiatum (dendrites) and stratum pyramidale (cell bodies) regions. Image intensities from stimulated regions (200–250 μm from the location of the stimulating electrode) were subtracted from unstimulated regions to give the difference in immunoreactivity. On rare occasion, the entire CA1 region was stained, and in these cases, the control region was measured in the CA3 area.

RESULTS

Correlation with postsynaptic action potentials

Staining for phospho-MAPK in sections from hippocampal slices was predicted to increase when stimulation typically effective in inducing LTP was delivered, because previous studies using immunoblots had shown increased phosphorylation of MAPK with this stimulation (English and Sweatt, 1996; Liu et al., 1999). Despite the significance of back-propagating dendritic action potentials to LTP (for review, see Linden, 1999; Paulson and Sejnowski, 2000), it is unknown whether action potentials are important for the activation of MAPK. We first tested whether the propagation of evoked population spikes across the CA1 subfield was accompanied by the appearance of staining for phospho-MAPK over a similar spatial area. Indeed, as the stimulation intensity increased, the greater the area of staining. The extent of staining across the CA1 subfield was significantly correlated with stimulation intensity ($p < 0.001$; Spearman's rank order correlation) (Fig. 1*A*). Similarly, an intensity that typically failed to evoke population spikes (but evoked field synaptic responses) (Fig. 1*B*, *60 μA*, ~500 μm from the stimulating electrode) also failed to induce staining in the same regions (Fig. 1*A*, *60 μA*). A "pathological" stimulation intensity range was not likely to be necessary for the staining, because the population spikes had not reached asymptotic levels at even 140 μA at the mid-CA1 recording site (Fig. 1*B*, *bottom panel*). We next tested whether blocking postsynaptic action potential spike generation and propagation with muscimol (10 μM), an agonist of the GABA-A receptors, blocked the staining for phospho-MAPK. Muscimol was found to completely block the staining increase across CA1 in slices stimulated with TBS at 140 μA ($n = 6$ slices; data not shown). Finally, the extent of staining induced with 140 μA of TBS stimulation was severely curtailed by 20 μM CNQX (124 ± 25.2 μm; $n = 3$ slices; data not shown), demonstrating that excitatory synaptic activity, and not electrical current per se, is responsible for the increase in phospho-MAPK staining.

Dependence on frequency of stimulation

With a stimulation intensity of 140 μA, TBS, as well as 100 Hz stimulation, induced intense staining for phospho-MAPK in hippocampal CA1. In many slices, distinct staining of the apical dendrites and cell bodies of CA1 pyramidal neurons could be identified (Fig. 2), although, in many instances, such structure was less obvious. This near-black staining is in stark contrast to the visible row of very lightly stained pyramidal cells in unstimulated slices, most evident at higher antibody concentrations (data not shown; but see Fig. 1*A*, *top*), indicating that the enzyme is maintained in a primarily unphosphorylated form in these neurons under control conditions.

In an effort to determine whether MAPK was activated under conditions not typically associated with LTP, we delivered the same number of pulses (120) at 1, 3, 5, and 10 Hz. As shown in Figure 2, stimulation given at 5 and 10 Hz was as effective as stimulation at LTP-inducing frequencies in both stratum radia-

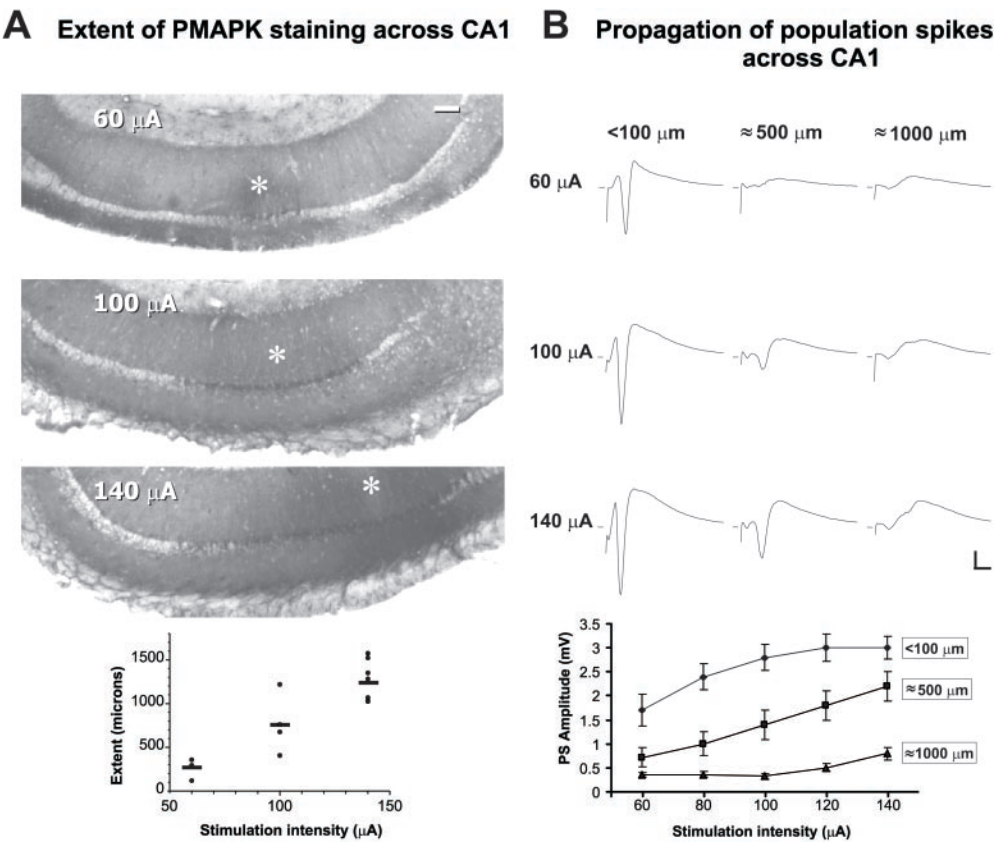

Figure 1. Extent of phospho-MAPK staining is related to the spread of population spikes across CA1. *A*, Staining for phospho-MAPK across CA1 was induced by theta-burst stimulation at three intensities, 60, 100, and 140 μA, and the largest distance between positive neuronal somata measured across CA1 (plotted at *bottom left*). The correlation between extent of staining and stimulation intensity was significant ($p < 0.001$; Spearman's rank order correlation; $n = 4$, 4, and 6 slices, for 60, 100, and 140 μA, respectively). Scale bar, 100 μm. *B*, Spread of population spikes across CA1 depends on intensities similar to those for inducing phospho-MAPK staining. At 60 μA, little spread beyond the immediate vicinity of the stimulating electrode was observed (*top row* of *traces*). With stimulation intensities of 100 and 140 μA, visible population spikes were observed 500 μm away (*second column* of *traces*). At 1 mm away from the stimulating electrode, population spikes were only discernable at and above 140 μA (*third column* of *traces*). Portions of the stimulus artifacts were removed for clarity of the figure. Calibration: 0.6 mV, 2.5 msec. Summary of population spike data are shown at the *bottom* ($n = 7$–9).

tum and stratum pyramidale regions. The increase in staining for phospho-MAPK was dependent, however, on stimulation frequency in that it was not activated with 1 Hz stimulation and was variably activated at 3 Hz (Fig. 2). The staining in response to 3 Hz occasionally reached levels of that at higher frequencies in individual cells but more often appeared in a lighter, perinuclear pattern and frequently failed to stain. At 1 Hz, only the neurons appearing in direct contact with the stimulating electrode, some apparent interneurons, and unidentified fibers were stained. These results demonstrate that, when controlled for intensity of stimulation and number of pulses, frequency is a determining factor in the activation of MAP kinase and that stimulation at 5 Hz results in MAPK phosphorylation, although LTP is not typically induced by this frequency in our preparation.

Dependence on route of calcium entry

Different patterns of gene expression induced with neuronal activity have been suggested to rely on the spatial selectivity of the distribution of NMDA receptors and VSCCs on dendrites and somas, respectively, for selective regulation of genes (Ginty, 1997; Hardingham et al., 1999). Those studies, however, had been performed on neuronal and non-neuronal cell cultures in which the precise spatial stimulation of postsynaptic neurons was not usually possible. Accordingly, we pursued the question of whether NMDA- and VSCC-dependent calcium increases could be resolved with the activation of MAPK using two different stimulation protocols (5 Hz and TBS). Interestingly, we found that 50 μM D-APV, an NMDA-receptor antagonist, was effective in blocking the effects of 5 Hz stimulation but appeared ineffective against the TBS-induced phospho-MAPK staining (Fig. 3). In contrast, we found that 20 μM nifedipine, which blocks L-type calcium channels, was ineffective in preventing phosphorylation of MAPK in stratum radiatum and pyramidale using either stimulation paradigm (Fig. 3). To get a significant blockade of the phospho-MAPK staining induced with TBS, a combination of APV and nifedipine was required (Fig. 3). APV and nifedipine together produced a slight additional decrease in the staining induced with 5 Hz. As was the case with the different frequencies, the staining in stratum pyramidale paralleled that observed in stratum radiatum, with no apparent selectivity for the dendritic or somatic fields of CA1. These data show that, although different stimulation protocols can differentially recruit voltage-dependent calcium channels, any spatial selectivity between the two sources of calcium was not apparent with staining for phospho-MAPK.

DISCUSSION

Using the phosphorylation of MAPK, the experiments described here were designed to test the degree to which spatially distinct sources of calcium and frequency of stimulation can modulate

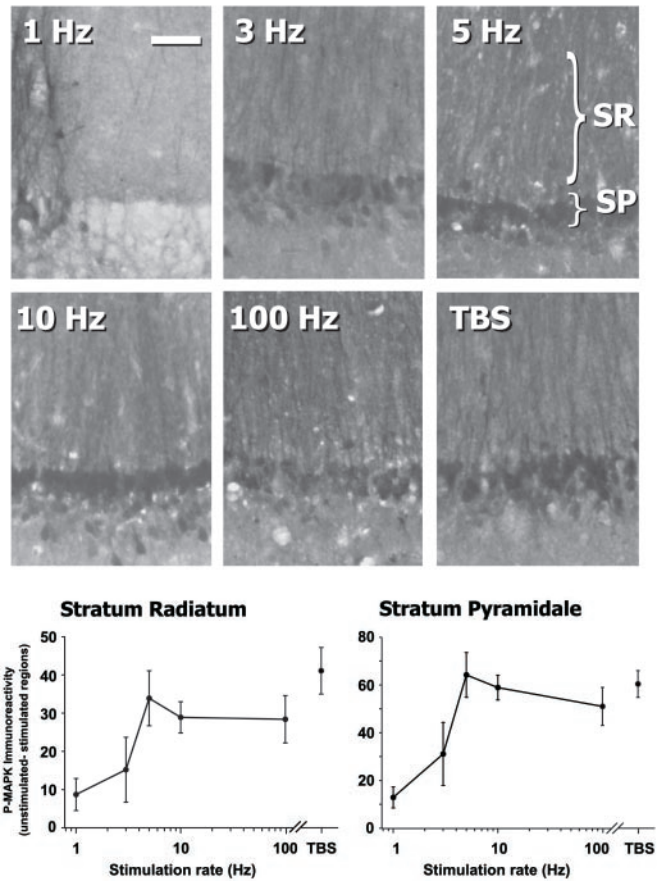

Figure 2. Staining for phospho-MAPK is frequency-dependent. Slices were stimulated for 120 pulses at 140 μA at the indicated frequencies. In all cases, positive staining in stratum radiatum (dendrites) paralleled staining in stratum pyramidale (somata). Stained neurons on the *left* of the *1 Hz panel* are cells near or contacting the stimulating electrode. Scale bar, 50 μm. n = 6, 6, 6, 8, 8, and 7 slices for 1, 3, 5, 10, and 100 Hz and TBS, respectively.

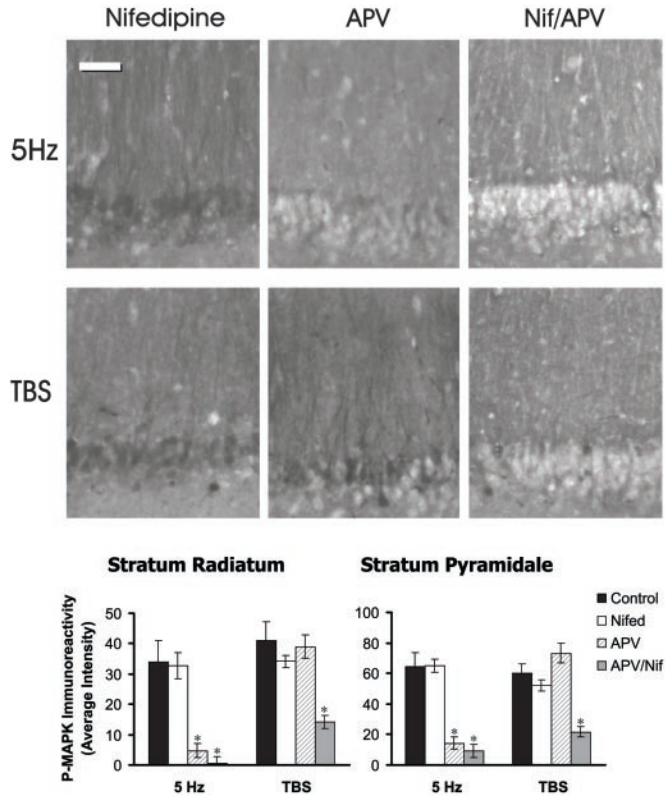

Figure 3. Phospho-MAPK staining induced with different stimulation displays distinct sensitivities to pharmacological blockade of calcium influx. Nifedipine did not reduce phospho-MAPK staining induced with either 5 Hz or theta-burst stimulation (n = 7 and 8 slices). APV significantly blocked staining induced with 5 Hz stimulation but not with TBS (n = 7 and 6 slices). When TBS was used for stimulation, significant reduction of phospho-MAPK staining was only observed when NMDA receptors and VSCCs were both blocked (n = 9 slices for TBS, 6 slices for 5 Hz). Staining in stratum radiatum paralleled staining in stratum pyramidale under all pharmacological conditions.

cellular signaling in somatodendritic compartments. We found that the staining across CA1 for phospho-MAPK required stimulation intensities that approximate the recruitment of postsynaptic action potentials. Supporting this assertion is that the extent of the staining across CA1 could be severely reduced by inhibiting excitatory synaptic transmission with CNQX and completely blocked by a GABA-A agonist, muscimol, which decreased postsynaptic spiking and voltage-dependent currents. We also found that, although staining induced with different frequencies differ in their sensitivity to NMDA and VSCC antagonists, the spatial distribution of the staining was distinguished by neither frequency nor drug condition. Because under all pharmacological and physiological conditions tested dendritic staining correlated with somatic staining, these data are most consistent with the idea of cell-wide increases in calcium, or other second messengers, leading to the activation of MAPK in a cell-wide manner.

Our pharmacological studies have yielded several unexpected results. First, we found that, although both 5 Hz and TBS can induce the phosphorylation of MAPK, only TBS recruits VSCCs to a degree that seems capable of substituting for NMDA receptor activation in inducing the phospho-MAPK stain. These findings show that calcium, through VSCCs, which may be necessary for transcription of some genes, is differentially recruited with theta-burst stimulation but not with 5 Hz stimulation to produce increases in phospho-MAPK staining. Because MAPK itself seems to be activated under both conditions, however, other kinases and/or messengers may be responsible for distinguishing pulses (activation of NMDA receptors) from bursts (NMDA receptors and VSCCs) to generate different immediate-early gene responses. Activation of calcium–calmodulin-dependent protein kinases, shown to recruit CREB-binding protein, are good candidates for such a role (Chawla et al., 1998; Hu et al., 1999).

A second unexpected finding was that phosphorylation of MAPK in the soma appeared to parallel phosphorylation in the dendrites. We find this especially intriguing in the case of APV blockade of NMDA receptors in which one might have expected a soma-only staining pattern. This was not the case, because APV blocked both dendritic and somatic staining when 5 Hz stimulation was used, indicating that NMDA receptor activation was sufficient to lead to staining in the cell bodies. Also unexpected was that neither dendrite nor soma compartments were reduced with APV when theta-burst stimulation was used, indicating that VSCC are recruited to a degree that induces staining, even in the dendrites. Greater intensities of stimulation induce larger synaptic responses and resulting action potentials because they recruit a larger number of afferent fibers converging on postsynaptic neurons. Thus, although it is certainly true that higher intensities would recruit a spatially larger afferent pool and synaptic activation, it is not likely to explain the observation that staining of

dendrites was not found without somatic staining. Usually, the dissociation tended to be the reverse; somatic staining in the absence of visible dendritic staining was sometimes observed at the margins of the stained areas, with the staining gradually decreasing in the most distal dendrites first. Our observations suggest that MAPK is not activated substantially in spatially discrete regions of the dendritic tree, but that it could instead be activated in a coordinated manner, cell-wide. This idea is consistent with our experiments showing a correlation of staining with the recruitment of action potentials and that action potentials can back-propagate into the apical dendritic tree (for review, see Linden, 1999).

Previous studies have investigated the activation of MAPK induced with electrical stimulation, but the range of control stimulus frequencies and intensities was limited [for example, 0.05 Hz (Impey et al., 1998) or 20 pulses at 20 Hz (Liu et al., 2000)]. The present findings are most compatible with the recent results of Winder et al. (1999), which correlated MAPK activation and LTP induction with stimulation that induces complex spikes (bursts of action potentials in CA1 neurons) (for review, see Lisman, 1997). Complex spikes evolved during 5 Hz stimulation and were facilitated by β-adrenergic receptor activation, which leads to the activation of PKA and MAPK-dependent signaling pathways (see also Thomas et al., 1998). Interestingly, both our study and that of Winder et al. (1999) showed that MAPK is activated by stimulus patterns that are not effective in inducing LTP, indicating that MAPK activation is not sufficient for LTP induction. Our present findings have extended the previous studies to indicate that somatic action potentials lead to the activation of MAPK via calcium influx requiring activation of L-type calcium channels and/or NMDA receptors.

In conclusion, different stimulus patterns lead to the activation of MAPK via different modes of calcium influx that are localized to different subcellular regions of the neuron. Although MAPK can be activated by several membrane receptors and types of neuronal excitation and can regulate a number of cellular substrates involved in synaptic plasticity, calcium influx through L-type calcium channels in response to somatic action potentials is most closely correlated with activation of MAPK in association with LTP.

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